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ARTICLE

3-(4-Methylfuran-3-yl)propan-1-ol: A White-Spotted Stinkbug (*Eysarcoris ventralis*) Repellent Produced by an Endophyte Isolated from Green Foxtail

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Stinkbug is a major rice plant pest in Asia. The extract of the culture filtrate of a fungus isolated from a green foxtail, *Setaria viridis* (L.) Beauv., was found to have a repellent effect on the white-spotted stinkbug, *Eysarcoris ventralis* (Westwood). The active principle was purified and isolated, and identified as 3-(4-methylfuran-3-yl)propan-1-ol (**1**) on the basis of spectroscopic data. Four acyl derivatives were prepared from **1** and assessed for repellent effect on the stinkbug; the acetyl derivative **2** was most effective.

KEYWORDS: *Eysarcoris ventralis*; *Setaria viridis*; repellent; endophyte; 3-(4-methylfuran-3-yl)propan-1-ol

INTRODUCTION

"Pecky rice" is the grain sucked by stinkbugs at the milky or pasty stage of rice and is partially or wholly stained, probably by bacteria or fungi that invade the grain at the sucking point. If a rice sample contains some pecky grains, both the quality and price are lowered according to the degree of contamination. In Japan there are about 10 species of stinkbugs that are regarded as major pests of rice; the most notorious among them are the sorghum plant bug, *Stenotus rubrovittatus* (Matsumura) (Heteroptera: Miridae), and the rice bug, *Leptocorisa chinensis* (Dallas) (Hemiptera: Alydidae) (*1*−*3*). The synthetic insecticides currently used to control these pests in Japan include neonicotinoids (dinotefuran, nitenpyram, and imidacloprid), organophosphates (fenitrothion and phenthoate), and pyrethroids (silafluofen and ethofenprox) (*4*). Applying insecticides too often not only produces insecticide-resistant insects, but disturbs the ecosystem. Using attractant or repellent to control these pests in place of insecticides is a promising alternative method of protecting the rice plant. Although there have been some reports on stinkbug attractant including a pheromone of the white-spotted spined bug *Eysarcoris parvus* (Uhler) (*5*), an attractant pheromone for the male rice bug *L. chinensis* (Dallas) (*6*, *7*), sex attractant pheromones of the sorghum plant bug *S. rubrovittatus* (Matsumura) (*8*), and an aggregation pheromone from *Eysarcoris lewisi* (Distant) (*9*), there have been no reports on stinkbug repellents.

In this study, we attempted to find a new and natural repellent among secondary metabolites produced by fungal endophytes. The endophytes live within plants and show no external sign of the infection, and thus their metabolites must not be toxic to plants. This symbiotic relationship is believed to provide a competitive advantage for the host plant, increasing tolerance to abiotic/biotic stresses such as drought, disease, and insect pests (*10*, *11*). This suggests that the endophytic metabolites may contain insect pest toxins or repellents. We used the stinkbug, *Eysarcoris ventralis* (Westwood), as a test insect because it is known to be a rice pest in paddy fields in Japan (*1*) as well as in upland rice fields in Asia (*12*). In our screening search, the extract of the culture filtrate of an endophyte isolated from a green foxtail was found to have a repellent effect. The active principle was isolated and identified as 3-(4-methylfuran-3-yl)propan-1-ol (**1**). In this paper, we report the repellent activity of compound **1** and its derivatives.

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MATERIALS AND METHODS

General Experimental Procedures. Solvents and reagents used in this study were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, unless otherwise noted. UV spectra were recorded with a Hitachi U-2001. NMR spectra were measured with a JEOL JNM-ECP 500 spectrometer. Chemical shifts were referenced to $CDCl_3$ (δ_H 7.26, δ_C 77.0). Mass spectra were obtained with a JEOL AX505HA (direct probe). In EIMS, the ionization voltage was 70 eV. In CIMS, the reaction gas was isobutane. Merck Kieselgel 60 F254 was used for the TLC. The TLC solvent was 30% (v/v) acetone in *n*-hexane.

Insects. The stinkbugs, *E. ventralis* (Westwood) (Heteroptera: Pentatomidae), were collected from some unoccupied grounds on the Koyama Campus of Tottori University from June to September 2008 and kept at 24−26 °C under an ambient photoperiod in Petri dishes with brown rice and distilled water.

Microorganism. The fungus used in this study was isolated, identified as follows, and deposited as NITE AP-796 at NITE Patent Microorganisms Depositary of National Institute of Technology and Evaluation (NITE), Japan. A green foxtail plant (*Setaria viridis* (L.) Beauv.) was collected on the Koyama Campus of Tottori University, and the leaves were treated with ethanol for 1 min and then 5% sodium hypochlorite for 1 or 3 min. The leaves were then rinsed with sterile water, cut into small pieces with a sterilized razor, and placed onto malt medium containing streptomycin (50 mg/L) in plastic Petri dishes (90 × 15 mm). The Petri dishes were incubated at 24 °C in darkness, and the fungi growing close to the leaves were transferred to malt slant medium. The secondary metabolite of one of the fungi had a repellent effect on *E. ventralis* (Westwood),

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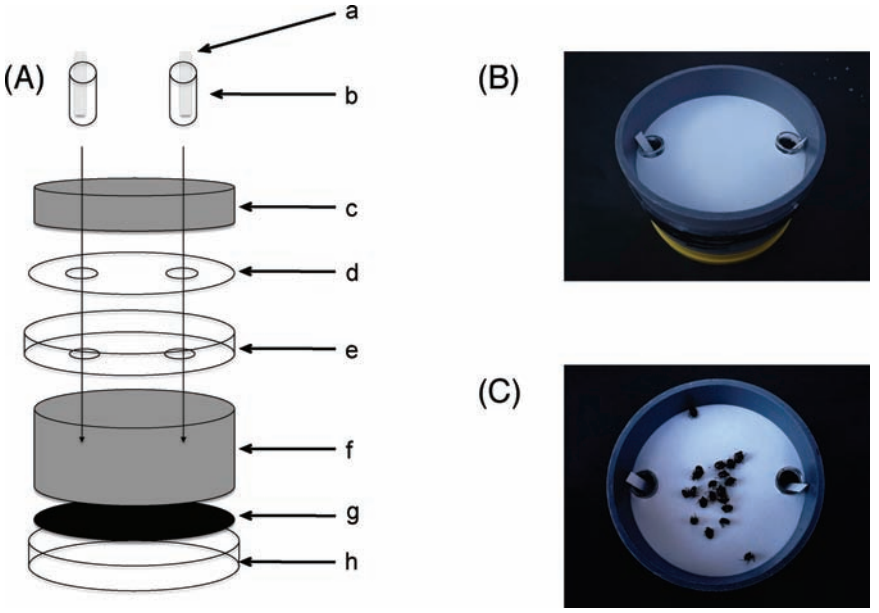


Figure 1. Repellent assay. (**A**) Structure of the assay apparatus: a, Advantec no. 2 filter paper (5 × 40 mm); b, glass bottle (12 × 35 mm); c, polyvinyl chloride pipe (88 × 17 mm); d, Advantec no. 2 filter paper (90 mm) with two holes (12 mm); e, lid (90 × 9 mm) of plastic Petri dish with two holes (12 mm); f, polyvinyl chloride pipe (88 × 42 mm); g, black paper (90 mm); h, lid (90 × 9 mm) of plastic Petri dish. (**B**) Assay apparatus. (**C**) Stinkbugs, *Eysarcoris ventralis* (Westwood), in the assay apparatus.

and the fungus was found to be similar to *Biscogniauxia atropunctata* in the sequence of their 18S rRNA gene: for analysis of the 18S rRNA sequence, the fungus was grown on PDA medium at 24 °C for 7 days. Mycelia were collected and disrupted with a pestle in the buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1% SDS). Genomic DNA was obtained with a Dneasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). The PCR used genomic DNA as the template and ITS2 and ITS5 as the PCR primers. Amplification conditions were 95 °C for 5 min, followed by 30 cycles of 95 °C for 15 s, 57 °C for 15 s, 72 °C for 30 s, and finally 72 °C for 5 min. The PCR products were purified with a Wizard SV Gel and PCR Clean-Up System (Promega) and directly sequenced. All of the sequencing reactions were done with a BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems), and the sequence determination was performed with an ABI PRISM 3130 sequencer.

Repellent Assay. The test sample was dissolved in an appropriate amount of acetone, and 50 μL of the solution was applied to a piece of filter paper (5 × 40 mm, no. 2, Advantec MFS, Inc.). For the control, only acetone (50 μL) was applied. These papers were dried and put into respective glass bottles (12 × 35 mm), which were connected to the assay apparatus (**Figure 1A**,**B**). Twenty stinkbugs, *E. ventralis* (Westwood), were introduced in the assay apparatus and left at 10 °C under light (**Figure 1C**). After 30 min, the number of bugs in each bottle was determined. The experiment was repeated four times. The repellent activity was computed from the formula

$$\text{repellent activity} = [100C/(C+T) - 50] \times 2$$

where C is the number of bugs in the control glass bottle and T is the number of bugs in the glass bottle containing the test compound. The average value and the standard error were obtained.

Isolation and Identification of Compound 1. The isolate of *Biscogniauxia* sp. was grown without shaking at 24 °C for 14 days in the dark in 500 mL conical flasks (50) containing liquid medium (200 mL/flask) composed of glucose (30 g/L), peptone (3 g/L), the extract from 50 g/L of malt, and water. Metabolites were extracted from the culture filtrate with 10 L of ethyl acetate three times after adjustment of the pH to 2.0 with HCl. The ethyl acetate solution was dehydrated over anhydrous sodium sulfate and concentrated to dryness to afford the extract (7.7 g). The extract was subjected to silica gel column chromatography (Daisogel IR-60, 62 × 720 mm), with 8 L each of 0, 5, 10, and 20% acetone in *n*-hexane. The fraction eluted with 10% acetone in *n*-hexane was concentrated to dryness to afford the active residue (660 mg). A portion (100 mg) of the residue was further purified by silica gel flash chromatography (Wakogel FC-40, 30 × 390 mm) with 200 mL (10 mL × 20) each of 10, 20, and 30% ethyl acetate in *n*-hexane saturated with water. Fractions 1−9 eluted with 20% ethyl acetate in *n*-hexane saturated with water were combined and concentrated in vacuo to afford the active compound (**1**, 59 mg) as colorless oil. When necessary, further purification by HPLC was carried out with a Cosmosil $5C_{18}$-AR column (Nacalai Tesque, 10 × 250 mm), 60% MeOH in water as the solvent, a flow rate of 1.0 mL/min, and monitoring at 250 and 280 nm (t_R 29 min): UV (MeOH) λ_{max} (log ε) 218 (3.93) nm; ^{1}H NMR and ^{13}C NMR data, see **Table 1**; EIMS, m/z (%) 140 (M^+, 14), 109 (8), 96 (100), 95 (55), 81 (8), 79 (8), 77 (6); HREIMS, m/z 140.08447 (calcd for $C_8H_{12}O_2$, 140.08373).

Esterification of Compound 1. Compound **1** (10 mg) was dissolved in pyridine (500 μL) and treated with acetic anhydride (500 μL) overnight at room temperature. The reaction mixture was poured into acidic water (pH 2.0) and the product extracted with ethyl acetate. The ethyl acetate solution was washed with 1 M sodium bicarbonate and brine, dried over anhydrous sodium sulfate, and concentrated. TLC purification of the ethyl acetate extract afforded compound **2** (8.9 mg): UV (MeOH) λ_{max} (log ε) 219 (3.40), 206 (3.57) nm; ^{1}H NMR and ^{13}C NMR data, see **Table 1**; EIMS, m/z (%) 182 (M^+, 9), 139 (2), 122 (100), 109 (14), 107 (9), 96 (62), 95 (100), 93 (38), 79 (38), 77 (13).

Compound **1** (9.5 mg) was dissolved in acetone (1 mL) and treated with pyridine (12 μL) and propionyl chloride (12 μL) overnight at room temperature. The workup procedure was the same as in the preparation of compound **2**. TLC purification gave compound **3** (7.6 mg): UV (MeOH) λ_{max} (log ε) 218 (3.69), 205 (3.61) nm; ^{1}H NMR and ^{13}C NMR data, see **Table 1**; CIMS, m/z (%) 197 (100).

Compound **1** (9.7 mg) was dissolved in acetone (1 mL) and treated with pyridine (23 μL) and butyryl chloride (29 μL) overnight at room temperature. The workup procedure was the same as in the preparation of compound **2**. TLC purification gave compound **4** (4.0 mg): UV (MeOH) λ_{max} (log ε) 215 (3.67), 205 (3.65) nm; ^{1}H NMR and ^{13}C NMR data, see **Table 1**; CIMS, m/z (%) 211 (100%).

Compound **1** (11.5 mg) was dissolved in acetone (1 mL) and treated with pyridine (19 μL) and benzoyl chloride (28 μL) overnight at room temperature. The workup procedure was the same as in the preparation of compound **2**. TLC purification gave compound **5** (7.4 mg): UV (MeOH) λ_{max} (log ε) 276 (3.31), 235 (4.21), 205 (4.05) nm; ^{1}H NMR and ^{13}C NMR data, see **Table 1**; EIMS, m/z (%) 244 (1), 139 (2), 122 (100), 105 (37), 104 (10), 96 (11), 95 (23), 93 (26), 79 (21), 77 (32).

Table 1. NMR Spectroscopic Data for Compounds **1**−**5** ($CDCl_3$)

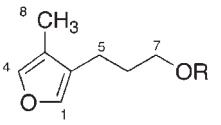
	1		2		3		4		5	
	δ_C	δ_H[a]	δ_C	δ_H[a]	δ_C	δ_H[a]	δ_C	δ_H[a]	δ_C	δ_H[a]
1	139.0	7.16 (1H, s)	139.1	7.15 (1H, s)	139.1	7.15 (1H, s)	139.1	7.14 (1H, s)	139.1	7.17[b] (1H, s)
2	124.8		124.2		124.3		124.3		124.2	
3	119.9		119.8		119.8		119.8		119.8	
4	139.6	7.16 (1H, s)	139.6	7.15 (1H, s)	139.6	7.15 (1H, s)	139.6	7.14 (1H, s)	139.6	7.19[b] (1H, s)
5	19.7	2.45 (2H, t, 7.8)	19.9	2.42 (2H, t, 7.7)	19.9	2.42 (2H, t, 7.6)	19.9	2.41 (2H, t, 7.8)	20.0	2.53 (2H, t, 7.6)
6	32.1	1.82 (2H, m)	28.2	1.88 (2H, m)	28.3	1.88 (2H, m)	28.3	1.87 (2H, m)	28.4	2.03 (2H, m)
7	62.4	3.71 (2H, t, 6.4)	63.9	4.11 (2H, t, 6.5)	63.7	4.11 (2H, t, 6.7)	63.6	4.10 (2H, t, 6.4)	64.3	4.37 (2H, t, 6.4)
8	8.0	1.97 (3H, s)	8.0	1.96 (3H, s)	8.0	1.95 (3H, s)	8.0	1.95 (3H, s)	8.1	1.98 (3H, s)
R			171.2		174.5		173.7		166.6	
			21.0	2.06 (3H, s)	27.6	2.33 (2H, q, 7.4)	36.2	2.28 (2H, t, 7.3)	130.3	
					9.2	1.15 (3H, t, 7.4)	18.5	1.66 (2H, m)	129.5[c]	8.05 (2H, d, 7.4)
							13.7	0.95 (3H, t, 7.3)	128.3[c]	7.45 (2H, t, 7.4)
									132.9	7.56 (1H, t, 7.4)

[a] Intensities, multiplicities, and *J* values (hertz) shown in parentheses. [b,c] Interchangeable.

RESULTS AND DISCUSSION

E. ventralis (Westwood) collected at our campus was used as the test insect. Through the course of our research we found that the most advantageous property of the insect is that when left under cool condition, they aggregate in a dark place. On the basis of this property the assay apparatus shown in **Figure 1A**,**B** was devised and used. The apparatus has two dark holes composed of glass bottles: a piece of filter paper (a) containing a test sample in one bottle (b) and a piece of filter paper (a) containing no sample in the other (b). Twenty stinkbugs were introduced onto the filter paper (d) on the lid of a Petri dish (e) as shown in **Figure 1C** and left at 10 °C under light for 30 min. During this time, each insect chose and entered a dark hole. Almost >70% of the insects were found in the bottles; the remainder stayed out of the holes. By comparing the respective number of insects found in the two holes, we could ascertain the repellent activity of the sample applied. By this assay, we screened secondary metabolites of the endophytes isolated from many grass weeds. In our screening search, the extract of the culture filtrate of an endophyte isolated from a green foxtail was found to have a repellent effect on the insect. The endophyte did not form spores in many trials, making identification difficult, but was found to be close to *B. atropunctata* (Schwein.) Pouzar (92% similarity) by a BLAST similarity search based on the sequence of its 18S rRNA gene.

The fungus was grown on malt extract medium without shaking at 24 °C for 14 days in the dark. The metabolites were extracted from the culture filtrate with ethyl acetate and purified by bioassay-guided chromatography to afford an active compound (**1**) in the yield of 39 mg/L. Compound **1** had the molecular formula $C_8H_{12}O_2$ from its HREIMS and NMR data, requiring three degrees of unsaturation. The ^{13}C NMR spectrum of **1** (**Table 1**) in $CDCl_3$ displayed eight carbon resonances: one owing to methyl, three to methylenes (one of which was oxygenated), two to sp^2 methines, and two to sp^2 quaternary carbons. Acetylation of **1** gave monoacetate **2**, confirming the presence of one hydroxyl in the molecule. The ^1H NMR spectrum of **1** (**Table 1**) indicated the presence of partial structures, $C-C(8)H_3$ and $C-C(5)H_2-C(6)H_2-C(7)H_2OH$. Taking the remaining carbons and protons, and their chemical shifts (δ_C 139.0 and 139.6; δ_H 7.16 and 7.16 for sp^2 methines, δ_C 119.9 and 139.0 for sp^2 quaternary carbons) into account, the 3,4-disubstituted furan ring in the molecule was suggested. The furan ring was also supported by its UV spectrum. Connecting these structural parts gave a structure, 3-(4-methylfuran-3-yl)propan-1-ol, for **1**. Assignments of the carbon resonances to carbon atoms of the furan ring were based on HMQC and HMBC data. 3-(4-Methylfuran-3-yl)propan-1-ol has been reported as a synthetic intermediate for alliacol A (*13*). On the basis of the spectroscopic and spectrometric data in the literature, compound **1** was completely identified as 3-(4-methylfuran-3-yl)propan-1-ol. It has never been reported as a natural product, and this is the first report of its production by a fungus.



1, R= H
2, R= $COCH_3$
3, R= $COCH_2CH_3$
4, R= $CO(CH_2)_2CH_3$
5, R= COC_6H_5

Figure 2. Structures of compounds **1**−**5**.

Table 2. Repellant Effect of Compounds **1**−**5** on White-Spotted Stinkbugs, *Eysarcoris ventralis* (Westwood)

	repellent activity (%) ± SE (*n* = 4)			
compound	0.003 μmol	0.01 μmol	0.1 μmol	1 μmol
1	−[a]	−	5.7 ± 4.6	89.3 ± 3.9
2	10.7 ± 3.2	7.3 ± 3.0	90.2 ± 3.2	−
3	−	20.5 ± 6.3	58.0 ± 5.4	89.3 ± 3.0
4	19.5 ± 6.3	−19.4 ± 0.8	51.8 ± 5.9	96.4 ± 2.3
5	−	−	5.3 ± 3.6	40.7 ± 6.8
naphthalene	−	40.7 ± 4.5	44.6 ± 6.3	80.4 ± 5.4

[a] −, not tested.

Four acyl derivatives (**2**−**5**; **Figure 2**) were prepared from compound **1**, and the repellent effects of compounds **1**−**5** were assessed by the assay using the white-spotted stinkbug (**Table 2**). A dose of 0.1 μmol of acetyl derivative **2** produced 90.2% repellency, which was more effective than the same dose of naphthalene. Doses of 0.1 μmol of propionyl and butyryl derivatives (**3** and **4**) produced moderate repellency comparable to the same dose of naphthalene. At the same dose amount, compound **1** and its benzoyl derivative (**5**) showed almost no effect. At a dose of 1 μmol, compound **1** and its propionyl and butyryl derivatives (**3** and **4**) showed high repellency (89.3, 89.3, and 96.4%, respectively), comparable to the same dose of naphthalene,

whereas benzoyl derivative (**5**) produced moderate repellency. Accordingly, the order of these compounds in the repellent activity was determined to be compound **2** > compound **3** = compound **4** > compound **1** > compound **5**. Interestingly, at a dose of 0.01 μmol, butyryl derivative (**4**) showed weak attractant activity, and this phenomenon was reproducible.

By this study we demonstrated that a metabolite produced by an endophyte showed repellent activity to a grass weed pest. Recently, Shiba and Sugawara (*14*) reported that loline alkaloids are generally observed in the highest concentrations in many *Neotyphodium*−grass symbiotic associations and are known to be toxic to insects; some *Neotyphodium*-infected grasses have enhanced resistance to the rice leaf bug, *Trigonotylus caelestialium*. If compound **1** is produced by the endophyte and accumulates in the foxtail plants, this symbiotic association enhances the resistance of the foxtail plants to the stinkbugs through the repellent effect by the compound. The role of this compound in the symbiosis is still unknown and remains to be investigated. As mentioned in the Introduction, "pecky rice" caused by stinkbugs is a serious agricultural problem in Japan. Acetyl derivative **2** could be an effective alternative chemical for controlling these pests, and experiments to assess its effect in pots and in fields are now being arranged.

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